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With generous support provided by the USAMRMC award, the interdisciplinary predoctoral training program in breast cancer research continues in its second year to provide rigorous training to students in a flexible, individualized context. This year, six trainees (three PhD students and three MD/PhD students) were selected from the current pool of students who were training in an area relevant to breast cancer research. In order to enhance their training and to supplement the already substantial graduate school curriculum, a variety of activities that had been developed last year were continued and expanded. These include a Breast Study Group, a Signal Transduction seminar series, Cancer Center seminars, and a Molecular Oncogenesis Research Colloquium. A graduate level course, Topics in Cancer Biology, seeks to incorporate the basic science of cancer with its clinical perspective. In addition, a number of journal clubs facilitate increased student and faculty interaction while giving students an opportunity to study selected topics in depth and to practice oral presentations. Thus, this program has been effective both in challenging students to undertake rigorous study in cancer research and in stimulating faculty to develop innovative strategies to understand the molecular mechanisms of tumorigenesis and to effectively intervene in the treatment of breast cancer.

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Stan Cannon 8/9/96

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PROGRESS REPORT SUMMARY- YEAR 2 (7/1/95-6/30/96)

INTRODUCTION

Mount Sinai's Breast Cancer Predoctoral Training Program (BCPTP) continues to provide rigorous training to predoctoral M.D./Ph.D. and Ph.D. candidates in an interdisciplinary and flexible context. Trainees are selected from the pool of students pursuing their advanced degree in cancer research, who have a specific interest in breast cancer. This report covers progress in year two (7/1/95-6/30/96) of this program.

BODY

PROGRESS OF TRAINEES

Jessica Feinleib (preceptor, Robert Krauss, Ph.D.)

Jessica Feinleib is working on a project studying tumor suppressor genes. Her lab recently isolated a potential tumor suppressor gene cdo whose sequence suggests that it is a cell adhesion molecule which is related to DCC. She is characterizing this new gene by constructing retroviral expression vectors containing this gene as well as determining focus forming activity as a result of expression of this gene in transfected rat fibroblast cells. She is also interested in studying potential roles that this gene may play in breast cancer and is utilizing RT PCR techniques as an initial step to answering this question.

Ms. Feinleib is also interested in studying the intracellular signaling that occurs within cells. In particular, she has been working on ras, one of the most potent oncogenes. By comparing the mRNA expression of many primary and secondary response genes in the ER-1-2/ras and ER-1-2 cell lines to control cell lines, one of which does grow in soft agar (PKC3-F4) and one of which does not grow in soft agar (PKC3-F4/ras), Ms. Feinleib seeks to understand the differences between anchorage-independent growth from oncogene induced gene expression. The primary response genes examined are fos-B, jun-B, c-jun, and c-myc and several secondary response genes. She finds that the ER-1-2 cell line does not express any of these genes while the non-transformed ER-1-2/ras expressing cell line does express them. This suggests that the expression of the primary and secondary genes examined are not sufficient for ER-1-2/ras cell line to acquire anchorage-independent growth. Results from these studies have been published in Molecular Carcinogenesis (Appendix A).

Maximilian Fonarev (preceptor, James Manfredi, Ph.D.)

Max Fonarev works on a project involving the regulation of the tumor suppressor activity of p53 by cyclin-dependent kinases. Specifically, he is studying the underlying mechanism by which overexpression of two cyclins, cyclin D1 and cyclin E, contribute to the oncogenic process in human breast cancer. The tumor suppressor protein p53 is phosphorylated by cyclin-dependent kinases and transcriptionally activates the gene encoding for a cyclin-dependent kinase inhibitor, p21. The hypothesis being

tested is whether cyclin overexpression exerts its oncogenic effects via inactivation of the tumor suppressor activity of p53, either by directly modifying the p53 by phosphorylation or by blocking the effects of its downstream target p21.

The initial approach has been to establish breast carcinoma cell lines which overexpress the various cyclins. He found that sustained cyclin overexpression is cytotoxic to this cell line. Therefore, he is now attempting to examine the effects of the overexpression of various cyclins in a transient assay system rather than in established clones. Co-transfection with a plasmid expressing the cell surface antigen CD20 will allow subsequent staining with a fluorescently tagged anti-CD20 antibody to distinguish transfection techniques with cells that lack p53 in order to determine the specific effects of cyclin overexpression. Mr. Fonarev is interested in studying p53 because mechanisms of regulating p53 may reflect mechanisms of oncogenesis.

Ulrich Hermanto (preceptor, Lu-Hai Wang, Ph.D.)
Ulrich Hermanto is working on a project that involves the study of intracellular signal transduction in breast cancer cells. Since growth factors and their receptors are known to play important roles in the mitogenicity and differentiation of normal cells and since various lines of evidence demonstrate that receptor protein tyrosine kinase overexpression and activation occur in various types of cancer cells, Mr. Hermanto screened a panel of breast tumor cell lines for various receptor protein tyrosine kinase expression, possible activation, as well as their downstream signaling molecules.

Several interesting results follow his initial study, begun last year with support from the Predoctoral training grant. His results confirm the reported overexpression of EGF receptor and erbB2/HER2 in certain breast cancer lines. No constitutive activation of the receptors was found. The expression of the IGF-1 receptor (IGFR) was found to be ubiquitous and moderately high in all cell lines examined and was elevated, relative to normal, in MCF-7, T47D, and BT20. The IGFR in all lines was responsive to stimulation by IGF-1 as measured by in in-vivo and in-vitro receptor tyrosine kinase autophosphorylation. He also shows that several known downstream signaling molecules for the IGFR were expressed and phosphorylated (i.e. p46 and p52 SHc). However, p66 SHc expression and phosphorylation were reduced in some cell lines. Other signaling molecules that were studied include Grb2, IRS-1, which were both overexpressed. From these results he speculates that overexpression of the IGFR and/or its downstream signaling mediators like IRS-1 and Grb2 could lead to enhanced sensitivity and response to IGF-1. He is continuing further investigation into the roles of IGFR signaling in breast cancer by transfecting several dominant negative mutant IGFR signaling molecules and assessing their effects.

Wei Li (preceptor, Xiangwei Wu, Ph.D.)
Wei Li is currently investigating the tumor suppressor gene p53 because it is known that mutations of p53 are among the most common alterations associated with breast cancers. The ability of the p53 protein to suppress tumor growth is attributed to two major biological processes induced by p53: cell cycle arrest and apoptosis. Mounting evidence shows that p53 induced apoptosis gives rather compelling effects on its

tumor suppressor function. However, still very little is known about the direct downstream signaling mediators of p53 leading to apoptosis. Also, the molecular basis of apoptosis and mechanism of apoptosis have not been clearly elucidated thus far.

In order to examine some of these questions, Ms. Li took advantage of the powerful strategy of mRNA differential display, and based on the established mouse fibroblast cell lines which undergo p53 mediated cell cycle arrest and apoptosis, she identified a putative new zinc finger transcription factor. From her preliminary results, she found that this gene product seems to be triggered not only during apoptosis but may be regulated in the normal cell cycle, most likely functioning as a G2/M checkpoint protein. The major goal of her thesis is to investigate the role and regulation of this gene product in the normal cell cycle as well as in p53 induced apoptosis.

Tara Santore (preceptor, Srinivas Iyengar, Ph.D.)
Tara Santore is pursuing research in the area of signal transduction, specifically attempting to determine if interactions between signaling pathways can be used to suppress expression of the transformed phenotype in mammary epithelial cells. Previous results from her laboratory have shown that interactions between the cAMP and MAP kinase pathways can be used to suppress transformation. Expression of activated Gas only modestly increased the cellular concentrations of cAMP but almost completely suppressed transformation of NIH-3T3 cells by H-ras. Since blockade of transformation by Gas could be achieved without raising cellular cAMP concentrations to harmful levels, it was thought that targeted implantation of the activated Gas may be a useful strategy for preventing the development of cancer in some tissues.

Ms. Santore has recently completed construction of an adenovirus vector containing a mutant Gas linked to a FLAG epitope, in order to facilitate its detection by an antibody to the epitope tag. This viral vector construct will be used to transfect a number of cell types in order to assess whether expression of activated Gas will result in suppression of colony formation in soft agar by several human breast cancer cell lines. At the same time, Ms. Santore will generate tumors in Nu/Nu mice by injection of these cell lines and determine if application of the recombinant adenovirus containing Gas results in tumor suppression.

SELECTION OF TRAINEES

Each applicant was required to submit an abstract of their research goals, a copy of their transcript, and a cover letter of support from their faculty preceptor.
The budget for year two provided partial stipend and tuition support for five trainees. This reflects the maximum number of trainees that could be supported based on the amount of funding provided. Applications were reviewed by members of the BCPTP Steering Committee (Appendix B). Members include the training grant Principal Investigator, Dr. Stuart Aaronson, and Dr. Miki Rifkin, an Associate Professor within the graduate school. Dr. Aaronson has first hand knowledge of all faculty research programs in cancer, as well as the academic qualifications of faculty members throughout the Medical School. Dr. Rifkin has intimate knowledge of the academic

qualifications of each prospective trainee. The qualifications of each trainee are outlined in Appendix C. The criteria used to select this years trainees were: (1) demonstration of academic achievement; (2) commitment to cancer research with an interest in breast cancer; (3) a detailed research proposal with both scientific merit and relevance to the study of breast cancer tumors; and (4) the academic qualifications of the preceptor. These selection criteria applied for both new and continuing applicants. In addition, applicants requesting an extension of their fellowship were also evaluated on the basis of their research progress.

Of the qualified applicants, the Steering Committee selected two new trainees, and three were carried forward from year one (Appendix C).

EVALUATION OF TRAINING PROGRAM

Each trainee has an advisory committee consisting of three faculty members chosen from the Molecular Basis of Disease and Cellular and Molecular Biological Sciences faculty. Selection of advisory committee members is tailored to the research and academic interests of each trainee. Trainees are required to meet with their advisory committee biannually. At these meetings, trainees review their research projects and summarize their educational progress including course work, journal clubs and seminars. The committee then prepares a written report summarizing the trainees' strengths and weaknesses, including suggestions for future directions. This report is given to each student, as well as submitted to the graduate school.

COURSES/SEMINARS/JOURNAL CLUBS

Several approaches have been utilized to stimulate student and faculty interest in breast cancer research. These include the development of a broad graduate training program in the Molecular Basis of Disease in which Cancer Biology is a major component. The first year course, to be initiated in the Spring of 1997, will include 14 lecture hours on cancer, and will cover an in-depth introduction to the molecular basis of cancer. This course is broadly advertised and provides faculty whose interests encompass breast cancer the opportunity to attract students to the program. As one concrete example of the focus on breast cancer, a major topic for the mandatory journal club for students enrolled in the first year of this multidisciplinary program will include a series on genes involved in DNA repair and their role in breast cancer. The number of faculty with an interest in cancer has expanded substantially in the first two years of the training grant. The Cancer Center presently has nine tenure track faculty with several additional recruitments projected in the next few months. This reflects a major commitment on the part of the Institution to develop and expand basic and translational research on cancer, and increases markedly the number of faculty with interests encompassing breast cancer.

Our philosophy is to encourage student interest in learning about cancer research and the remarkable progress in our understanding of the underlying molecular mechanisms as well as provide a diverse array of forums which highlight research efforts and specific unsolved questions in the area of breast cancer. We believe that if a student with high academic potential initiates studies in a research area of

fundamental importance to cancer, participation in our multidisciplinary conferences, seminars, and colloquia which emphasize breast cancer topics will stimulate and encourage career development toward research directly pertinent to this disease.

Topics in Cancer Biology

This fifteen week graduate level course was developed in 1995 as an elective for students interested in cancer research. In three modules the following topics are covered: Oncogenes (module 1); Tumor Suppressor Genes (module 2); and Tumor Biology (module 3). While not required, training program participants are encouraged to select this elective, or at a minimum attend the following lectures:

- Growth Factors and Cancer
- p53 (Parts 1 and 2)
- WT1/BRCA1
- Mechanisms of Carcinogenesis
- Microscopy of Selected Tumors

Journal Clubs

Cancer research journal clubs are available through the Molecular Basis of Disease Program and Cellular and Molecular Biological Science programs. Included in this year's weekly journal club offerings were signal transduction, cell adhesion and integrins, and cell pathology. Training program participants are required to regularly attend at least one of these cancer related journal clubs. The journal club format requires that students critically review and present their analyses of recent journal articles.

Seminars, Colloquia and Conferences

Cancer Center Research Seminar

Sponsored by the Ruttenberg Cancer Center, this monthly seminar provides faculty, postdoctoral fellows, trainees and students with the opportunity to present and receive input on their "works in progress". Senior and junior faculty from the Molecular Basis of Disease and Cellular and Molecular Biological Sciences programs, as well as the Director of the Cancer Center, provide critical analysis and guidance. Each BCTP trainee is given the opportunity to present their work at this seminar.

Cancer Center Invited Lecture Series

Organized by the Ruttenberg Cancer Center, this series sponsors nationally recognized scientists to lecture on cancer research topics such as angiogenesis, cell adhesion, tumor suppressor genes, cell cycle, and oncogenes. Over twenty three lectures were sponsored during year two (Appendix D). Of these, five seminars were offered as part of the BCPTP program.

- Molecular Detection of Human Cancer, presented by David Sidransky, M.D.
- Regulation of Structure and Function of the p53 Tumor Suppressor Protein, presented by Carol Prives, Ph.D.
- Ubiquitination of Cell Cycle Proteins is Involved in the G1 Checkpoint, presented by Michele Pagano, M.D.

- Control of Mammary Carcinoma Cell Proliferation by Vitamin A, presented by David Talmage, Ph.D.
- Genes Involved in Breast Cancer, presented by Alexander Kamb, Ph.D.

Molecular Oncogenesis Research Colloquia

This monthly colloquia is designed to promote interdepartmental interaction and collaboration among the Medical School's basic science laboratories working in the areas of oncogenes, suppressor genes and oncogenesis. During each session two laboratories present a short update of their research findings. Through this colloquia, students are exposed to the broad array of cancer research. BCPTP trainees are encouraged to participate in these sessions.

Signal Transduction Seminar Series

This monthly research colloquium of signal transduction and molecular oncogenesis brings together scientists from twelve to fifteen laboratories sharing this common research interest. The goal of the colloquium is to promote collaboration and sharing of ideas and reagents with the perspective of developing program project initiatives along the course. The topics discussed encompass various aspects of cytokines, growth factors, growth factor receptors, mediated normal and oncogenic signal transduction, oncogenes, tumor suppressor genes and their roles in cell growth, cell transformation, apoptosis, as well as in human malignancy.

Community Medicine Seminar Series

The Department of Community Medicine offers grand rounds, monthly seminars, and brown bag lunches, covering various topics including environmental carcinogenesis, environmental epidemiolgy, and women's health.

RECRUITMENT OF NEW TRAINEES/PROGRAM ADVERTISEMENT

Each year the program is advertised within the medical school through dissemination of a written announcement to faculty throughout the Molecular Basis of Disease and Cellular and Molecular Biology Sciences Programs, and to each faculty member participating in this training grant. New training grant faculty members are enlisted if their research interests are directed toward breast cancer. In addition, the Cancer Center sponsors an orientation in the fall to provide students with the opportunity to meet with faculty informally and learn about initiatives in breast cancer, as well as alert them to opportunities in training as part of the BCPTP.

FACULTY COLLABORATION

The Breast Cancer Study Group, sponsored by the Ruttenberg Cancer Center, fosters collaboration between clinician scientists, basic science researchers and psychosocial researchers, with the major goal of translating basic research advances for clinical practice. More than ten Departments and Centers, are involved in this multidisciplinary working group. The study group meets quarterly and trainees are required to participate in these forums. As an example of one such meeting, Dr. Jonathan Licht, Department of Medicine, discussed basic research advances with respect to the BRCA1 breast cancer suppressor gene, and Dr. Christine Eng, Human

Genetics, described efforts to establish genetic testing for breast cancer within the Institution.

One objective measure of the success of this training program in fostering interest in breast cancer has been the increase in grant submissions by faculty for projects that are breast cancer related. For example, our scientists submitted twenty research grants, and three postdoctoral fellowship grants in response to the July 1996 Department of Defense Breast Cancer Research Program Announcement. This reflects both the increasing number of cancer research oriented faculty in the Cancer Center, and the much greater interaction and collaboration among cancer research oriented faculty within the entire Mount Sinai Medical Center fostered by our cancer research seminars/colloquia, and courses as well as the training grant itself.

CONCLUSIONS

The various seminars, courses, colloquia that were organized last year and modified this year have continued to stimulate interest among the faculty and students in an interdepartmental approach to the study of breast cancer. In Year Three these activities will be expanded to meet more frequently and to include more faculty as interest in this program grows. As new faculty are recruited to the Cancer Center and to other departments in the School of Medicine, they will be made aware of these activities and invited to participate. These activities will also serve to stimulate research collaboration among the Mount Sinai faculty in the area of breast cancer, and will hopefully lead to new insights into the molecular mechanisms responsible for the disease.

Therefore, in review, the objectives for year two have been met and these objectives will be expanded during year three of the training program, in accordance with the Statement of Work provided in the original grant proposal.

The second year of this program supported five trainees. During the next budget period, the number of trainees supported by this grant will be six. Faculty have already submitted names of suitable candidates for the trainee slots and the Steering Committee has met and selected the most qualified students for these slots.

REFERENCES

Trainee Publications: Feinleib, J.L. and Krauss, R.S. (1996) Dissociation of ras oncogene-induced gene expression and anchorage-independent growth in a series of somatic cell mutants. Molecular Carcinogenesis 16:139-148 (1996).

MOLECULAR CARCINOGENESIS 16:139–148 (1996)

Dissociation of *ras* Oncogene-Induced Gene Expression and Anchorage-Independent Growth in a Series of Somatic Cell Mutants

Jessica L. Feinleib and Robert S. Krauss[1]

Department of Biochemistry, Mount Sinai School of Medicine, New York, New York

The mechanism or mechanisms by which *ras* oncogenes induce morphological transformation and anchorage-independent growth are poorly understood but are thought to involve stable alterations in gene expression. We previously described a genetically dominant, mutant rat fibroblast cell line (ER-1-2) that is resistant to *ras*-induced anchorage-independent growth. We now describe a cell line derived from ER-1-2 cells, termed ER-1-2T, that has apparently sustained a second, dominant mutation that conferred on these cells the ability to form colonies in soft agar. Analysis of these and control cell lines demonstrated that deregulation of many of the genes commonly associated with the transformed phenotype could be dissociated from anchorage-independent growth. After infection with a *ras*-expressing retrovirus, both control and ER-1-2 cell lines constitutively expressed elevated levels of the c-*jun*, *junB*, *fosB*, c-*myc*, collagenase, ornithine decarboxylase, osteopontin, stromelysin, cathepsin L, and insulin-like growth factor 1 genes. These data indicate that signaling events downstream of *ras* were largely intact in ER-1-2 cells and that the defect in these cells lies either on a pathway separate from those that control stable, *ras*-mediated expression of these genes or at a point in the cell-division cycle distinct from those that control expression of the genes. In contrast, only c-*jun*, *jun*B, c-*myc*, and ornithine decarboxylase were expressed at a significantly elevated level in ER-1-2T cells. Thus, deregulated expression of the genes analyzed was not sufficient for anchorage-independent growth. Furthermore, deregulation of most of them was also not necessary.

Key words: *ras* oncogene, somatic cell mutant, anchorage-independent growth, gene expression

INTRODUCTION

ras is a major transducer of extracellular signals that regulate proliferation and differentiation of cells. Furthermore, members of the *ras* family of oncogenes are among the most frequently mutated genes in human cancers [1–3]. Introduction of mutant, activated *ras* genes into cultured rodent fibroblasts causes neoplastic transformation of these cells, including alterations in cell shape and adhesiveness and induction of anchorage-independent growth. This latter property correlates almost invariably with tumorigenicity [4,5] and is a useful in vitro end-point for the dissection of the aggressive growth properties of transformed cells. The specific mechanisms by which ras effects such changes in cells are not completely obvious, but it is widely believed that at least some of these changes occur as a result of stable alterations in gene expression [6]. Thus, the identification of genes whose deregulated expression causes the transformed phenotype is pivotal to the molecular elucidation of the aggressive growth properties of tumors. Numerous genes that are ordinarily expressed in a tightly regulated temporal manner when quiescent cells are stimulated to reenter the cell cycle (i.e., primary and secondary response genes [7]) are often constitutively expressed in *ras*-transformed cells, but the individual roles of these genes in mediating the deranged growth control seen in such cells are not well established [6].

It has recently become evident that ras exerts its pleiotropic effects via multiple and interactive pathways. Genetic studies on the yeast *Schizosaccharomyces pombe* indicate the existence of at least two ras effectors, byr2 and scd1 [8]. In mammalian cells, White et al. [9] have demonstrated that ras has at least two functions that can contribute to transformation, one of which is mediated via the established ras effector raf-1. Very recently, it was found that the small GTPase rac plays an essential role in mediating ras' ability to transform rat fibroblasts and, furthermore, that this role is apparently independent of ras-mediated activation of raf and the mitogen-activated protein kinase cascade [10]. The pathway or pathways by which ras feeds into rac are, however, currently unclear. The results of biochemical studies complement these genetic data in that at least five mammalian proteins are known to interact physically with ras in a man-

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MT, metallothionein; DMEM, Dulbecco's modified Eagle's medium; ODC, ornithine decarboxylase; IGF-1, insulin-like growth factor 1.

ner consistent with some effector function: raf, phosphatidylinositol-3-OH kinase, the GTPase-activating protein/neurofibromin family proteins, ral guanine nucleotide dissociation stimulator, and rin1 [11–20].

It is striking that at least three of these potential effectors have been implicated in signaling to the nucleus and induction of gene expression. For example, it is well established that raf can activate gene expression by stimulating the mitogen-activated protein kinase cascade, which results in phosphorylation of such transcription factors as Elk-1 and c-myc [21–23]. Additionally, a constitutively activated form of phosphatidylinositol-3-OH kinase and a deletion mutant of GTPase-activating protein that encodes the amino-terminal SH2 and SH3 domains each can activate the c-*fos* promoter in transient transfection assays [24,25]. Finally, the ras effector rac can stimulate the stress-activated protein kinase/c-jun amino-terminal kinase cascade, leading to phosphorylation of such transcription factors as c-jun and ATF-2 [26,27]. It is clear, then, that activated ras can signal through multiple effectors, many of which have the potential to influence gene expression in a manner that may contribute to maintenance of the transformed phenotype.

Determining which ras-induced pathways and which target genes contribute to different aspects of the transformed phenotype is essential to a productive understanding of neoplastic transformation. We have developed an interesting somatic cell genetic system to gain insight into this process. We previously described two mutant Rat 6 fibroblast cell lines that were isolated by a novel selection procedure [28]. It was reported earlier by Hsiao et al. [29] that Rat 6 embryo fibroblasts that expressed high levels of both the T24 Ha-*ras* oncogene and protein kinase C (PKC) β1 were killed by exposure to the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). It was postulated that overstimulation, by TPA, of signaling pathways controlled by ras and PKC leads to cytotoxicity and, further, that survivors of such toxicity might include somatic cell mutants that were also resistant to the transforming effects of these two genes [28]. Such mutants were selected by treatment of a Rat 6 cell subclone that overexpressed PKCβ1 and harbored a heavy metal-inducible metallothionein (MT) I–T24 Ha-*ras* construct (PKC3-A5 cells) with both TPA and $ZnSO_4$ [28]. The mutant cell lines that were isolated retained high-level expression of PKCβ1 but no longer expressed the MTI-T24 Ha-*ras* construct, even in response to $ZnSO_4$ and other inducers. Additionally, the mutants also had defects in induction of endogenous *MTI* and *MTII* genes. Critically, however, these mutant cell lines were resistant to the induction of anchorage-independent growth by infection with retroviruses that contained the v-Ha-*ras*, v-*src*, or v-*raf* oncogenes. Furthermore, this resistance to transformation was dominant in somatic-cell hybrids. Interestingly, while the mutants failed to grow in soft agar in response to these retroviral oncogenes, they mounted a morphological response (i.e., rounded-up and refractile appearance and highly disordered growth on tissue-culture plastic) that closely resembled that of fully transformation-competent, wild-type cells. The morphological alteration was particularly pronounced with v-Ha-*ras*. Thus, these mutant cell lines were able to dissociate *ras*-mediated signals that control morphological alteration from those that control anchorage-independent growth and permit, therefore, analysis of these two phenotypes individually [28,30]. Because these revertant cell lines arose at single-gene–mutation frequencies, we hypothesized that a single mutation was responsible for the two easily observable properties of these cells: failure to induce the MTI-T24 Ha-*ras* construct and endogenous *MTI* and *MTII* genes and failure to proliferate in soft agar in response to certain oncogenes. It seems reasonable to assume that genes that are critical for anchorage-independent growth might, like the *MTI* and *MTII* genes, also be repressed in these cells. We report here studies on one of the mutant cell lines, ER-1-2, and another variant of these cells that reacquired the ability to grow in soft agar.

MATERIALS AND METHODS

Cell Culture and Soft-Agar Assays

All of the cell lines used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD) with 10% bovine calf serum (HyClone Laboratories Inc., Logan, UT), and the cultures were fed every 3 d with fresh medium. Soft-agar assays were performed in a single well of a six-well dish as follows: 10 000 cells of the designated cell line were suspended in 2 mL of 0.3% Noble agar (Difco Laboratories Inc., Detroit, MI) in DMEM containing 10% calf serum and overlaid on a layer of 2.5 mL of 0.5% agar in the same medium. The cells were then fed twice a week by overlaying them with 2 mL of 0.3% agar in DMEM with 10% calf serum. The plates were incubated at 37°C for 2 wk and then stained for 3 d at 37°C with p-iodonitrotetrazolium violet vital stain (Sigma Chemical Co., St. Louis, MO). The colonies were then counted with a low-power microscope.

PKC Assays

PKC activity was assayed as described by Housey et al. [31]. Briefly, PKC was partially purified by passing detergent extracts of whole cells through a DEAE Sephacel column and eluting with 0.5 M NaCl. Total PKC activity was assayed in the presence or absence of phosphatidylserine plus Ca^{2+} for 10 min at 30°C with a synthetic peptide of the epidermal growth factor receptor (RKRTLRR) as a substrate. The specific activity was then calculated as the amount

of phospholipid-dependent incorporation of ^{32}P into the peptide per mg of protein per min, minus the background values.

Nucleic Acid Analyses

Total RNA was extracted from whole-cell lysates by the guanidine thiocyanate/cesium chloride protocol, as previously described by Krauss et al. [32]. Fifteen micrograms of RNA per sample were then electrophoresed through agarose/formaldehyde gels and transferred onto Hybond N nylon membranes (Amersham Corp., Arlington Heights, IL) in 10× standard saline citrate. The membranes were then crosslinked with ultraviolet light, hybridized to ^{32}P-labeled probes, washed, and exposed to x-ray film [32]. The probes used included c-*jun* [33], *jun*B (American Type Culture Collection, Rockville, MD), c-*fos* [33], osteopontin [34], stromelysin [35], ornithine decarboxylase (*ODC*) [36], collagenase [37], cathepsin L [38], and insulin-like growth factor 1 (*IGF-1*) (a gift from A. Efstradiadis, Columbia University).

Southern blot analysis of genomic DNA was performed as described previously [39].

Somatic-Cell Hybridization

Puromycin- and histidinol-resistant derivatives of ER-1-2, ER-1-2T, and R6-C1 were constructed by infection with the retroviral vectors pBabePuro [40] and pMSVhis [41] and selection in medium containing 2.5 μg/mL puromycin and 10 mM histidinol, respectively. Before cell fusion, 2.5×10^6 cells of each singly drug-resistant cell line were seeded into a 6-cm dish and cultured overnight. After 24 h, the cells were treated with 45% polyethylene glycol 1000 for 30 s, washed five times with fresh medium, and allowed to grow overnight in nonselective medium. The next day the cells were split 1 to 5 into medium containing 2.5 μg/mL puromycin and 10 mM histidinol. Replicate plates of cells that were not exposed to polyethylene glycol were used as controls for the efficacy of the double-selection medium. These cells all died within 3 d, at which time the double drug–resistant cells were pooled and assayed for growth in soft agar.

RESULTS

Generation and Characterization of the ER-1-2T Cell Line

A summary of the key properties of the cell lines used in this study is presented in Table 1. As described in the Introduction and shown in Table 1, the ER-1-2 cell line overproduces PKCβ1 but does not form colonies in soft agar and is resistant to *ras*-induced anchorage-independent growth. To our surprise, when 1×10^7 ER-1-2 cells were injected into nude mice, tumors arose after a latent period of 10 wk. The cells from one such tumor were cultured, and a cell line, designated ER-1-2T, was established. As seen in Figure 1, the morphology of ER-1-2T cells closely resembled that of the untransformed parental cell line, ER-1-2, although the ER-1-2T cells grew to a somewhat higher density. For comparison purposes, ER-1-2 cells infected with a v-Ha-*ras*–expressing retrovirus (i.e., ER-1-2/*ras* cells) are also shown. These latter cells were highly morphologically transformed but did not proliferate in soft agar [28].

The development of tumors from ER-1-2 cells in nude mice could have been due to intrinsic, but very weak, tumorigenicity of these cells, despite their anchorage-dependent phenotype in culture. Alternatively, the tumors could have arisen as the result of the clonal outgrowth of an ER-1-2 cell variant that had sustained a transforming somatic mutation. To distinguish between these possibilities, the ability of ER-1-2T cells to proliferate in soft agar was tested. If the tumors arose because ER-1-2 cells were intrinsically tumorigenic, ER-1-2T cells would, like the pa-

Table 1. Cell Lines Used in This Study

Cell line	Exogenous genes	Morphological transformation*	Colony formation in soft agar†	References
R6-C1	*neo*	–	–	Housey et al. [31]
C1-D3	*neo, hph*	–	–	Krauss et al. [28]
PKC3-F4	*neo, hph, PKCβ1*	–	+	Krauss et al. [28]
PKC3-F4/*ras*	*neo, hph, PKCβ1*, v-Ha-*ras*	+	++++	Krauss et al. [28]
PKC3-A5	*neo, hph, PKCβ1*, MT-T24 c-Ha-*ras*	+	++++	Krauss et al. [28]
ER-1-2	*neo, hph, PKCβ1*, MT-T24 c-Ha-*ras* (silent)	–	–	Krauss et al. [28]
ER-1-2/*ras*	*neo, hph, PKCβ1*, MT-T24 c-Ha-*ras* (silent), v-Ha-*ras*	+	–	Krauss et al. [28]
ER-1-2T	*neo, hph, PKCβ1* MT-T24 c-Ha-*ras* (silent)	–	++++	This study

*–, grew as a flat, ordered monolayer; +, rounded, refractile appearance with disordered growth on culture plates.
†–, no growth; +, formation of microscopic colonies in 3 wk; ++++, formation of macroscopic colonies in 2 wk.

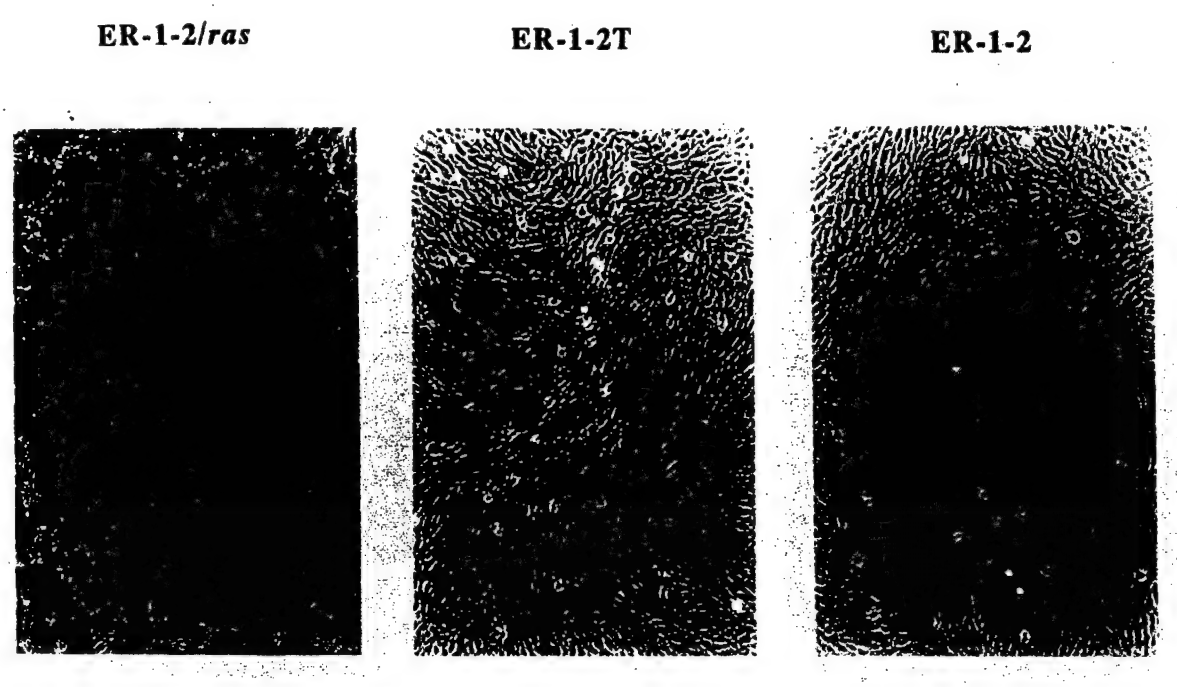

Figure 1. Photomicrographs of the ER-1-2, ER-1-2T, and ER-1-2/*ras* cell lines. Cells were grown to confluence in 10-cm plates in DMEM with 10% calf serum and then photographed. Magnification, 40×.

rental line, be anchorage dependent in culture; conversely, if ER-1-2T cells represent a truly transformed variant of ER-1-2 cells, they should proliferate in soft agar. As shown in Table 2, ER-1-2T cells formed colonies in soft agar very efficiently, at rates comparable to those of PKC3-A5 cells (the fully transformed cells from which ER-1-2 cells were derived). Furthermore, injection of 5×10^6 ER-1-2T cells into nude mice produced tumors 1 cm in diameter in 14 d, a time at which a similar number of ER-1-2 cells had not produced even a palpable mass (data not shown). Thus, the ER-1-2T cell line appears to be an anchorage-independent, tumorigenic clonal variant derived from the ER-1-2 cell line rather than an expanded subpopulation of it. It is interesting that the ER-1-2T cells did not display significant evidence of morphological transformation yet were anchorage independent. This is the opposite of ER-1-2/*ras* cells, which were grossly morphologically altered but were strictly anchorage dependent.

In light of the data presented above, the most likely explanation for the development of tumors from ER-1-2 cells is that a somatic mutation occurred in a single cell, conferring on this cell the transformed phenotype. Two types of mutation that could result in the transformed phenotype can be envisaged. The first is simple back mutation, i.e., the mutation that generated ER-1-2 cells from PKC3-A5 cells reverted to wild type. Alternatively, a second, forward mutation that resulted in anchorage-independent growth could have occurred. A distinctive property of ER-1-2 cells is that they no longer expressed the transfected MTI-T24 Ha-*ras* gene that is responsible for the transformed phenotype of the parental line, PKC3-A5 ([28] and see Introduction). ER-1-2 cells also show defective induction of the endogenous *MTI* gene in response to $ZnSO_4$ and other inducers, and this repression of MT promoters appears to have occurred as a result of the same mutation that conferred on these cells the transformation-resistant phenotype [28]. We predicted that if a back mutation to wild type had occurred in the ER-1-2T line, expression of the MTI-T24 Ha-*ras* and *MTI* genes would be restored, whereas if a second mutation had occurred these genes might still be repressed. Therefore, to determine which type of mutation was responsible for generating the ER-1-2T cell line, we

Table 2. Growth of Cell Lines in Soft Agar

Cell line	Colony formation (%)*	Average colony diameter (mm)
PKC3-A5	20.0	0.7
C1-D3	0	–
ER-1-2	0	–
ER-1-2T	49.1	0.35

*Ten thousand cells of the indicated type were grown in 0.3% agar for 2 wk, at which time the colonies were counted and colony-forming efficiency calculated. The values shown are the averages of triplicate determinations that differed by no more than 10%. The data shown are representative of at least four experiments for each cell line.

tested whether these cells had regained expression of the transfected MTI-T24 Ha-*ras* and endogenous *MTI* genes. Northern blots of RNA prepared from a variety of cell lines grown with or without 100 μM $ZnSO_4$ were hybridized to either T24 Ha-*ras* or *MTI* probes. Figure 2 demonstrates that the control cell line, PKC3-A5, exhibited basal and $ZnSO_4$-inducible expression of the MTI-T24 Ha-*ras* construct, whereas neither the ER-1-2 nor ER-1-2T displayed any T24 Ha-*ras* expression. The C1-D3 and PKC3-F4 cell lines (Table 1), which were not transfected with this construct, also had no signal and served as negative controls. It should be noted that the ER-1-2T cell line retained multiple unrearranged copies of the transfected MTI-T24 Ha-*ras* construct, as determined by Southern blot analysis of genomic DNA with a T24 Ha-*ras* probe (data not shown). The expression pattern of the endogenous *MTI* gene was similar to that of the transfected MTI-T24 Ha-*ras* construct (Figure 2). The C1-D3, PKC3-F4, and PKC3-A5 cell lines all exhibited basal (and robust $ZnSO_4$-inducible) expression of this gene. In contrast, both the ER-1-2 and ER-1-2T cell lines lacked basal expression of *MTI* and displayed an obviously diminished ability to express *MTI* in response to $ZnSO_4$. These data clearly indicate that the expression of genes controlled by MT promoters in the ER-1-2T cell line was similar to the defective expression observed in ER-1-2 cells and, therefore, that ER-1-2T cells had not regained normal regulation of these genes. These RNA analyses thus ruled out the possibility that the ER-1-2T cell line arose as the result of a simple back mutation that regenerated the PKC3-A5 cell line. Rather, the data strongly indicate that a second, additional mutation had occurred in ER-1-2T cells. Furthermore, this putative second mutation conferred anchorage-independent growth on these cells in the presence of the original mutation in the ER-1-2 cell line that dictates resistance to v-Ha-*ras*–, v-*src*–, and v-*raf*–mediated, anchorage-independent growth.

We next tested ER-1-2T cells for expression of the exogenous *PKCβ1* cDNA that was present in the grandparental (PKC3-A5) and parental (ER-1-2) cell lines. Previous studies established that ER-1-2 cells expressed levels of PKCβ1 enzyme activity that were roughly equivalent to those of PKC3-A5 cells ([28] and see Introduction). Northern blotting studies demonstrated that the ER-1-2T cell line produced an amount of *PKCβ1* mRNA similar to that produced by the PKC3-A5 and ER-1-2 lines (data not shown). Partially purified extracts of C1-D3, PKC3-A5, ER-1-2, and ER-1-2T cells were then assayed for PKC activity. As shown in Table 3, ER-1-2T cells expressed a level of activity that was very similar to that of ER-1-2 cells and about 15- to 20-fold higher than that of the control C1-D3 cells, which do not harbor the

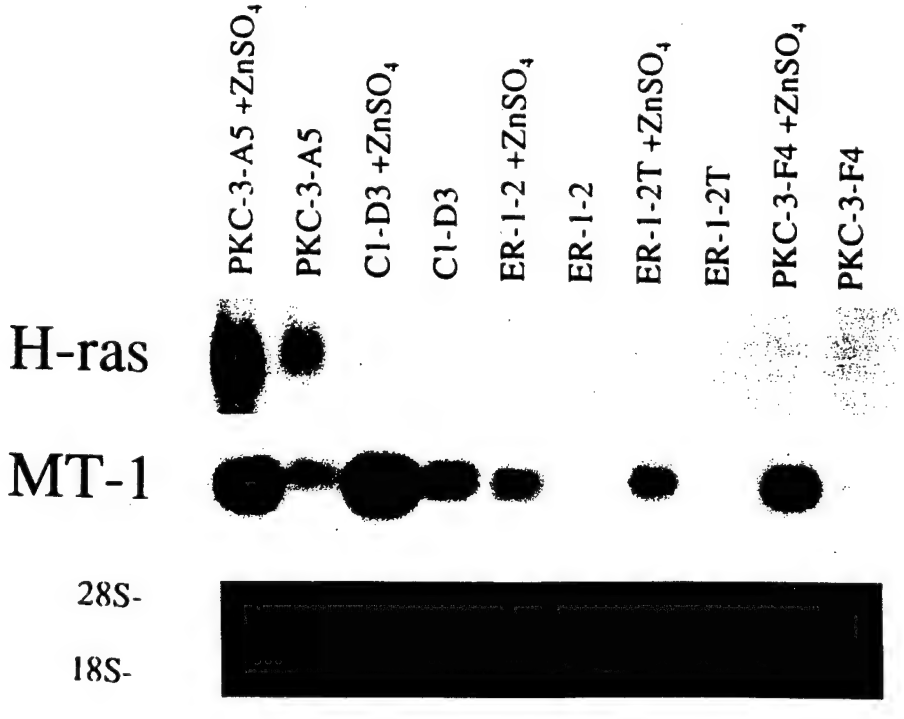

Figure 2. Northern blot analyses of the exogenous mRNA expression of MTI-T24 Ha-*ras* and endogenous *MTI* genes in various cell lines. Fifteen micrograms of total cellular RNA was fractionated on agarose/formaldehyde gels, transferred to nylon membranes, and hybridized to the indicated ^{32}P-labeled probes. The ethidium bromide-stained gel, featuring the 18S and 28S rRNA bands, is shown as a loading control. See text for details.

Table 3. PKC Activity in C1-D3, PKC3-A5, ER-1-2, and ER-1-2T Cell Lines

Cell line	PKC activity (nmol/min/mg)*	Fold increase over PKC activity in C1-D3 cells
C1-D3	83	–
PKC3-A5	2430	29.3
ER-1-2	1560	18.8
ER-1-2T	1250	15.1

*PKC was partially purified from each of the indicated cell lines as described in Materials and Methods. PKC specific activity was defined as the amount of phospholipid-dependent ^{32}P incorporation into the synthetic peptide substrate (RKRTLRR) per mg of protein per min. The values shown are the averages of duplicate determinations that differed by less than 10% and are representative data from two independent experiments performed on two separate extracts.

PKCβ1 cDNA. Alterations in expression or activity of the exogenous *PKCβ1* gene therefore did not play a role in the acquisition of the transformed phenotype by ER-1-2T cells.

We previously demonstrated that the oncogene-resistant revertant phenotype of ER-1-2 cells is dominant over the transformed phenotype in somatic-cell hybridizations [28]. This suggests that ER-1-2 cells arose because of a dominant mutation. To test whether the transformed phenotype of ER-1-2T cells was dominant or recessive to the untransformed phenotype of ER-1-2 cells, this same technique of somatic-cell hybridization was applied. Puromycin-resistant derivatives of the ER-1-2 and ER-1-2T cell lines were each fused to histidinol-resistant derivatives of the ER-1-2 and R6-C1 cell lines. R6-C1 cells are an untransformed subclone of the original Rat 6 cell line ([31] and Table 1). After fusion and double-drug selection, the hybrids were tested for their ability to form colonies in soft agar (Table 4). Fusion of ER-1-2 cells either to themselves or to R6-C1 cells resulted in hybrids that, as expected, could not proliferate in soft agar. In contrast, fusion of ER-1-2T cells to ER-1-2 or R6-C1 cells yielded hybrids that grew in soft agar as efficiently as ER-1-2T cells alone. The "retransformed," anchorage-independent phenotype of the ER-1-2T cell line was therefore dominant over the revertant, oncogene-resistant phenotype of the parental cell line, ER-1-2. This suggests that the second, additional mutation that occurred in ER-1-2T cells is also dominant and that it is capable of overriding the effects of the first mutation.

Expression of Primary and Secondary Response Genes in a Series of Somatic-Cell Mutants

As mentioned in the Introduction, it is important to dissect out the transformation-induced genes that contribute to various aspects of the transformed phenotype. Gene expression analyses of the ER-1-2T cell line, in conjunction with our previously described panel of cell lines, offers an opportunity to do so in a set of mutants in which morphological transformation is dissociated from anchorage-independent growth. As controls we used PKC3-F4 cells ([28] and Table 1), a line that overexpresses PKCβ1, is minimally transformed, and responds to expression of the v-Ha-*ras* oncogene with full morphological alteration and anchorage-independent growth. ER-1-2 mutant cells, in contrast, also overexpress PKCβ1 but respond to v-Ha-*ras* only with morphological alteration [28]. Finally, the ER-1-2T cell line had (presumably by means of a second mutation) acquired the anchorage-independent growth phenotype within the ER-1-2 mutant genetic background but was not significantly morphologically transformed. We analyzed two classes of genes known to be deregulated by expression of the *ras* oncogene: primary response genes, i.e., genes that are rapidly induced in G_0-synchronized cells in response to serum or growth factors and secondary response genes, i.e., genes induced with delayed kinetics after such cells are stimulated. Total RNA was isolated from confluent cultures of cells that had not been fed with fresh medium for 48 h and was analyzed by northern blotting techniques. Figure 3 shows that the primary response genes *fosB, junB,* c-*jun,* and c-*myc* were each expressed at detectable levels in PKC3-F4 cells and were expressed at constitutively elevated levels in PKC3-F4/*ras* cells. These same genes were also expressed at constitutively elevated levels in ER-1-2/*ras* cells, despite the inability of these cells to proliferate in an anchorage-independent manner. In contrast, the ER-1-2T cell line overexpressed only the c-*jun* and c-*myc* genes at levels comparable to those of ER-1-2/*ras* and PKC3-F4/*ras* cells. The level of *junB*

Table 4. Growth in Soft Agar of Somatic-Cell Hybrids Derived From Various Cell Fusions

Hybridization number*	Cells expressing puromycin resistance	Cells expressing histidinol resistance	Colony-forming efficiency in soft agar (%)†
1	ER-1-2T	ER-1-2	36.4
2	ER-1-2T	R6-C1	44.3
3	ER-1-2	ER-1-2	0.1
4	ER-1-2	R6-C1	0.1

*The hybridization number refers to the fusion of the two cell types listed in the following two columns. After fusion the hybrid cells were selected with 2.5 μg/mL puromycin and 10 mM histidinol and then seeded into soft agar. The values shown are the averages of triplicate determinations that differed by no more than 10% and are representative of two independent experiments.
†The hybrids were grown in 0.3% agar for 2 wk, at which time the colonies were counted and colony-forming efficiency calculated.

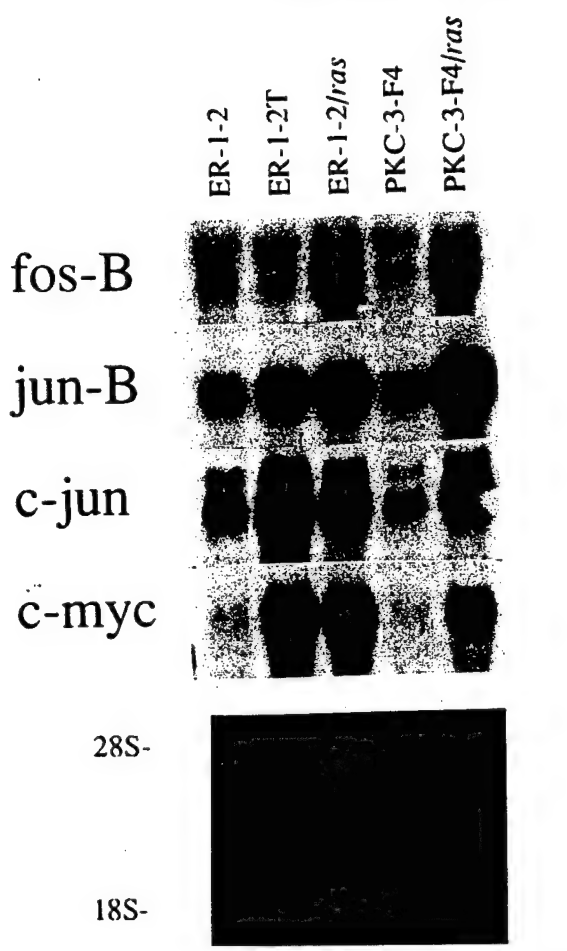

Figure 3. Northern blot analysis of the expression of a panel of primary response genes in various cell lines. Fifteen micrograms of total cellular RNA was fractionated on agarose/formaldehyde gels, transferred to nylon membranes, and hybridized to the indicated ^{32}P-labeled probes. The ethidium bromide-stained gel, featuring the 18S and 28S rRNA bands, is shown as a loading control.

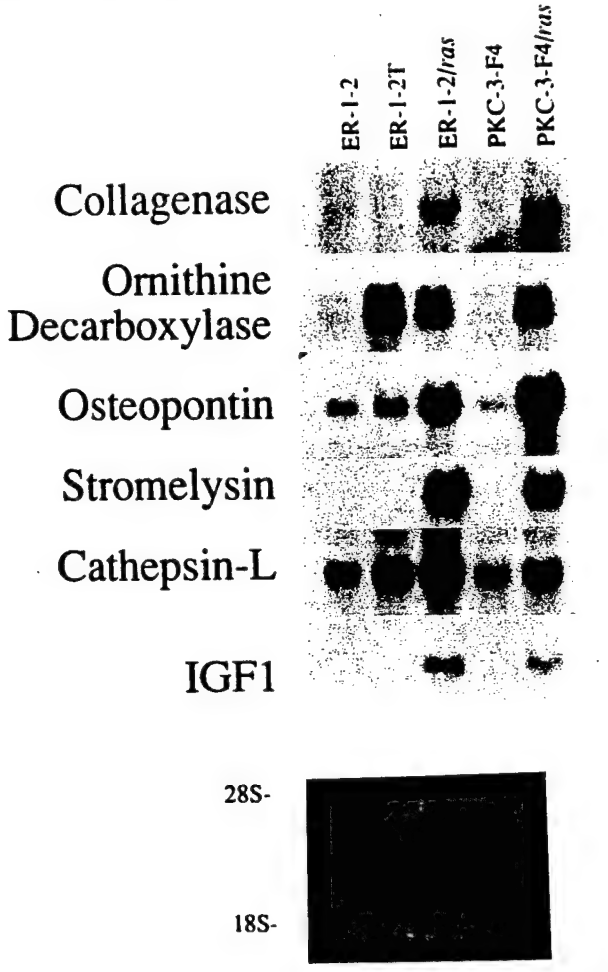

Figure 4. Northern blot analysis of the expression of a panel of secondary response genes in various cell lines. Fifteen micrograms of total cellular RNA was fractionated on agarose/formaldehyde gels, transferred to nylon membranes, and hybridized to the indicated ^{32}P-labeled probes. The ethidium bromide-stained gel, featuring the 18S and 28S rRNA bands, is shown as a loading control.

mRNA found in ER-1-2T cells was intermediate to the levels in PKC3-F4 and PKC3-F4/*ras* cells, suggesting partial deregulation of this gene. Thus, deregulated expression of at least four primary response genes in response to *ras* was not sufficient to induce ER-1-2 cells to grow in soft agar, whereas overexpression of *fosB* was not necessary for anchorage-independent growth of ER-1-2T cells. A fifth primary response gene, c-*fos*, was expressed at barely detectable levels in all five cell lines (data not shown).

A similar analysis was performed on the secondary response genes collagenase, *ODC*, osteopontin, stromelysin, cathepsin L, and *IGF-1* (Figure 4). As with the primary response genes, all six secondary response genes were expressed at constitutively elevated levels in PKC3-F4/*ras* cells relative to PKC3-F4 cells. Similar to the expression of the primary response genes, expression of each of the secondary response genes was also deregulated in ER-1-2/*ras* cells, despite their anchorage-dependent phenotype. Finally, of the secondary response genes examined, only the *ODC* gene was expressed at significantly elevated levels in ER-1-2T cells. Thus, widespread deregulation of primary and secondary response genes was not sufficient for anchorage-independent growth of ER-1-2/*ras* cells, whereas only a small number of changes in gene expression (in four of 11 genes examined) accompanied anchorage-independent growth in ER-1-2T cells.

DISCUSSION

This paper describes studies on the ER-1-2 mutant rat fibroblast cell line. These cells are genetically resistant to *ras*-induced anchorage-independent growth yet still undergo transformation-related morphological alterations in response to this oncogene [28]. The resistance of the ER-1-2 cell line to anchorage-inde-

pendent growth is dominant in somatic-cell hybrids [28], yet we report here the isolation of a variant cell line derived from ER-1-2 that had acquired the ability to form colonies in soft agar. This variant cell line, designated ER-1-2T, was derived from a tumor that developed after a long latent period after injection of a large number of ER-1-2 cells into a nude mouse. Two principal lines of evidence indicate that ER-1-2T cells arose as a result of a second, presumably extragenic, mutation that conferred anchorage-independent growth on these cells in the presence of the original ER-1-2 cell mutation that mediates resistance to the *ras* oncogene. First, a property that is closely correlated with resistance to *ras* in ER-1-2 cells, i.e., repression of the transfected MTI-T24 Ha-*ras* and endogenous *MTI* genes, was retained in ER-1-2T cells. Previous data indicate that a single mutation in ER-1-2 cells is probably responsible for both resistance to *ras* and defective expression of these genes [28]. Thus, continued repression of the MTI-T24 Ha-*ras* and *MTI* genes in ER-1-2T cells suggests that the gene originally mutated in ER-1-2 cells was still present and functioning as a mutant allele. Second, the anchorage-independent growth phenotype of ER-1-2T cells was dominant over the transformation-resistant phenotype of ER-1-2 cells in somatic-cell hybridizations. The most logical explanation for these observations is that one ER-1-2 cell sustained a second, transforming, somatic mutation and that this cell expanded clonally to give rise to the tumor from which the ER-1-2T cell line was cultured. The gene in which this transforming mutation occurred is not known but is clearly not *ras*, *src*, or *raf*, each of which failed to induce anchorage independence in ER-1-2 cells [28]. The dominant nature of the ER-1-2T gene suggests that this gene could be cloned by gene transfer technology, for example, by transfection of genomic DNA from ER-1-2T cells into ER-1-2 cells, and then selection for cells that grow in soft agar. We are currently attempting such experiments.

It is also noteworthy that ER-1-2T cells acquired the ability to proliferate in soft agar without becoming obviously morphologically transformed. Oncogenes like *ras* and *src* activate multiple signaling pathways that result in both morphological alterations and deregulated growth control. The pathways controlled by these oncogenes that are responsible for morphological changes were intact in the ER-1-2 cell line, despite this line's resistance to anchorage-independent growth ([28] and see Figure 1). The fact that ER-1-2 cells were susceptible to oncogene-induced morphological alteration and ER-1-2T cells were not suggests that the transforming gene in the latter cells deregulated a pathway or pathways that are selectively involved in the control of cell proliferation. The reciprocal phenotypes of ER-1-2/*ras* cells, which were morphologically altered but did not grow in soft agar, and ER-1-2T cells, which were anchorage independent but minimally morphologically transformed, are strong evidence that these two properties of transformed cells are controlled independently and can be dissociated genetically. Somatic-cell mutants derived from NRK cells that have a phenotype similar to that of ER-1-2T cells were recently reported by Assoian and coworkers [42].

The precise mechanism or mechanisms by which ras induces anchorage-independent growth are poorly understood. ras acts through multiple effectors and complex signaling pathways to effect neoplastic transformation of cells, and several of these effectors and pathways have been demonstrated to regulate gene expression (see Introduction). The development of the ER-1-2T cell line offered an attractive opportunity to analyze gene expression events in a series of somatic-cell mutants that showed very specific alterations in anchorage-independent growth properties. A total of five primary response genes (c-*jun*, *junB*, c-*fos*, *fosB*, and c-*myc*) and six secondary response genes (collagenase, *ODC*, osteopontin, stromelysin, cathepsin L, and *IGF-1*) were investigated. Ten of these 11 genes (i.e., all but c-*fos*) were constitutively expressed or overexpressed in control cells that were transformed by v-Ha-*ras*, and this pattern of gene deregulation was fully intact in ER-1-2 cells that expressed this oncogene. Thus, constitutive expression of these genes was not sufficient for ER-1-2/*ras* cells to proliferate in an anchorage-independent manner. Furthermore, this observation suggests that the signaling pathways downstream of *ras* that govern stable expression of these genes were largely intact in ER-1-2 cells. The defect in ER-1-2 cells therefore seems to lie either on a pathway separate from those that control stable expression of these genes or at a point in the cell-division cycle other than the temporal site of action of these pathways. It should be noted that the signaling pathways that control the transient induction of these genes in growth factor–stimulated cells may not be the same as those that govern their stable expression in transformed cells. Nevertheless, the *ras*-mediated pathways that do result in stable expression of these genes appear to be unaffected in ER-1-2 cells, despite these cells' inability to proliferate in soft agar.

Intriguingly, ER-1-2T cells displayed deregulation of only a small subset of these genes. Among the primary response genes, c-*jun*, *junB*, and c-*myc* but not *fosB* were clearly expressed at elevated levels in ER-1-2T cells; among the secondary response genes only *ODC* was significantly deregulated. Thus, the complete deregulation of the early response gene program that accompanies transformation by *ras* was neither sufficient (for ER-1-2/*ras* cells) nor necessary (for ER-1-2T cells) for anchorage-independent growth. It is remarkable that none of the three genes that encode proteases (collagenase, stromelysin, and cathepsin L) were substantially deregulated in ER-1-2T cells. Overexpression of these and other protease-

encoding genes is frequently observed in transformed and tumor cells [6]. Although we cannot rule out the possibility that ER-1-2T cells produce excessive quantities of these proteases in the absence of significant alterations in mRNA expression, the data reported here strongly indicate that their deregulation is not strictly required for anchorage-independent growth or tumorigenesis. This does not imply that these proteases are unimportant in tumorigenesis; their role in tumor invasion and metastasis is well established [43]. Nevertheless, it is apparent that cells can exhibit a profound deregulation of growth control without deregulation of at least three such proteases. Continued analysis of the series of mutants described in this paper should yield further insights into which *ras*-mediated signaling and gene expression events are necessary and sufficient for specific aspects of the transformed phenotype.

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APPENDIX B
BCPTP Steering Committee Members

Stuart A. Aaronson, M.D.	Ruttenberg Cancer Center
Srinivas R. V. Iyengar, Ph.D.	Pharmacology
Edward Johnson, M.D., Ph.D.	Pathology
Robert Krauss, Ph.D.	Biochemistry
Jonathan Licht, M.D.	Medicine
Mary Rifkin, Ph.D.	Molecular Biology
Lu-Hai Wang, Ph.D.	Microbiology
Mary Wolff, Ph.D.	Community Medicine

APPENDIX C
BCPTP Year 2 Trainees

Trainees Carried Forward From Year One

Maximilian Fonarev
Molecular Biology (Ph.D.), Year Three
Preceptor: James Manfredi, Ph.D.
B.S. in Physics, 1992, Moscow Phy. Tech.
GRE's: V-340, Q-770, A-640

Ulrich Hermanto
Microbiology (M.D./Ph.D.), Year Four
Preceptor: Lu-Hai Wang, Ph.D.
B.A. in Chemistry, 1992, Boston University
MCAT's: Vrb-11, Phy-10, Wrt-P, Bsc-12, TOT-33
Undergraduate GPA: 3.52

Tara Santore
Biochemistry (Ph.D.), Year Four
Preceptor: Srinivas Iyengar, Ph.D.
B.S. in Biology, 1990, St. Frances College
GRE's: V-460, Q-640, AN-610, ADV-660
Undergraduate GPA: 3.94

New Trainees Year Two

Jessica Feinleib
Molecular Basis of Disease (M.D./ Ph.D.), Year Four
Preceptor: Robert Krauss, Ph.D.
B.A. in Biology, 1992, University of Chicago
MCAT's: Vrb-9, Phy-9, Wrt-0, Bsc-11, TOT-29
Undergraduate GPA: 3.00

Wei Li
Molecular Basis of Disease (Ph.D.), Year Two
Preceptor: Xiangwei Wu, Ph.D.
B.S. in Biology, 1989, Xinjiang University
M.S. in Molecular Biology, 1992, Fudan University
GRE's: V-580, Q-800, AN-700, TOE-597

APPENDIX D

Topics in Cancer Biology
Course Outline

Module I. Oncogenes

Lecture 1.	Oncogenes defined.	Lu-Hai Wang, Ph.D.
Lecture 2.	The ras oncogene family.	Andrew Chan, Ph.D.
Lecture 3.	Growth factors and cancer.	Stuart Aaronson, M.D.
Lecture 4.	Oncogene activation and transcription factors.	Jon Licht, M.D.
Lecture 5.	Chromosomes and cancer.	Vesna Najfeld, Ph.D.

Module 2. Tumor Suppressor Genes

Lecture 1.	Introduction and pRb-Part 1	Jon Licht, M.D.
Lecture 2.	pRb-Part 2	Jim Manfredi, Ph.D.
Lecture 3.	p53-Part 1	Jim Manfredi, Ph.D.
Lecture 4.	p53-Part 2	Xiangwei Wu, Ph.D.
Lecture 5.	Cdk inhibitors	Jim Manfredi, Ph.D.
Lecture 6.	WT1/BRCA1	Jon Licht, M.D.
Lecture 7.	Neurofibromatosis	Jim Manfredi, Ph.D.
Lecture 8.	Genetic model for colorectal tumorigenesis/APC	Steve Itzkowitz, M.D.
Lecture 9.	DCC/hereditary colon cancer	Steve Itzkowitz, M.D.
Lecture 10.	Mismatch repair	Zhen-Qiang Pan, Ph.D.

Module 3. Tumor Biology

Lecture 1.	Intro. to course & Cancer and Development	Stave Kohtz, Ph.D.
Lecture 2.	Metastasis and Angiogenesis I	Liliana Ossowski, Ph.D.
Lecture 3.	Metastasis and Angiogenesis II	Liliana Ossowski, Ph.D.
Lecture 4.	Apoptosis	Xiangwei Wu, Ph.D.
Lecture 5.	Immunosurveillance and cancer	Karen Zier, M.D.
Lecture 6.	Mechanisms of Carcinogenesis	Rob Krauss, Ph.D.
Lecture 7.	Mechanisms of Viral Carcinogenesis	Stave Kohtz, Ph.D.
Lecture 8.	Microscopy of Selected Tumors	David Burstein, Ph.D.
Lecture 9.	Molecular Strategies for Cancer Therapy	Stuart Aaronson, M.D.

LECTURE SERIES SEMINARS - 7/1/95 - 6/30/96

NAME OF SPEAKER	AFFILIATION	SEMINAR DATE	SEMINAR TITLE
Gennaro D'Urso, Ph.D.	Imperial Cancer Research Fund, London, UK	September 11, 1995	"A role for the DNA replication initiation complex in the checkpoint coupling S phase to mitosis in fission yeast."
Arthur J. Lustig, Ph.D.	Memorial Slone-Kettering Cancer Center	October 6, 1995	"Telomere dynamics in yeast: Mechanisms regulating heterochromatization and recombination."
Jeffrey B. Stavenhagen, Ph.D.	Princeton University	October 10, 1995	"Yeast telomeres from the inside out."
Albert B. Reynolds, Ph.D.	St. Judes Children's Research Hospital	October 23, 1995	"Identification of a new catenin: The tyrosine kinase substrate P120^{CAS} associates with E-cadherin complexes."
Kiran Chada, Ph.D.	Univ. of Medicine & Dentistry of New Jersey Robert Wood Johnson Medical School	October 27, 1995	"The pygmy gene: Role of HMGI-C in development and tumorigenesis."
Sheila Thomas, Ph.D.	Fred Hutchinson Cancer Research Center	November 6, 1995	"Genetic and biochemical interactions of Csk and Src family kinases and their potential role in organization of the cytoskeleton."
Ulla M. Hansen, Ph.D.	Dana-Farber Cancer Institute	November, 8, 1995	"Transcriptional and structural counterpoint between histone H1 and chromosomal protein HMG-14."
William Meikrantz, Ph.D.	Harvard School of Public Health	November 13, 1995	"Apoptosis and the cell cycle."
Carol L. Prives, Ph.D.	Columbia University	December 19, 1995	"Regulation of structure and function of the p53 tumor supressor protein."
Bernd R. Seizinger, M.D., Ph.D.	Bristol-Myers Squibb Princeton University	January 30, 1996	"Tumor suppressor genes and their signaling pathways: novel targets for mechanism-based drug discovery?"
David Sidransky, M.D.	Johns Hopkins University School of Medicine	February 6, 1996	"Molecular detection of human cancer."

LECTURE SERIES SEMINARS - 7/1/95 - 6/30/96

continued....

NAME OF SPEAKER	AFFILIATION	SEMINAR DATE	SEMINAR TITLE
Filippo Giancotti, Ph.D.	NYU Medical Center	February 16, 1996	"Mechanisms of integrin mediated signal transduction."
Elizabeth Yang, Ph.D.	Washington University School of Medicine	February 26, 1996	"BAD: A regulator of cell death."
Carol L. Prives, Ph.D.	Columbia University	March 12, 1996	"Regulation of structure and function of the p53 tumor supressor protein."
Napoleone Ferrara, M.D.	Genentech, Inc.	March 29, 1996	"The biology of VEGF."
Michele Pagano, M.D.	Mitotix, Inc.	April 11, 1996	"Ubiquitination of cell cycle proteins is involved in the G1 checkpoint."
David Talmage, Ph.D.	Columbia Univèrsity	May 8, 1996	"Control of mammary carcinoma cell proliferation by vitamin A."
Leonard H. Augenlicht, Ph.D.	Albert Einstein College of Medicine	May 13, 1996	"Gene expression and structure in human colon cancer."
Vishva M. Dixit, M.D.	University of Michigan Medical School	May 28, 1996	"Identification of components of the cell death pathway."
Alexander Kamb, Ph.D.	Myriad Genetics, Inc.	June 3, 1996	"Genes involved in breast cancer."
Rick Cerione, Ph.D.	Cornell University-College of Veterinary Medicine	June 5, 1996	"CDC-42-A GTP-binding protein that is involved in everything."
Nickolas Papadopoulos, Ph.D.	Johns Hopkins Oncology Center	June 10, 1996	"Genetic basis of hereditary non-polyposis colorectal cancer."
Noel Bouck, Ph.D.	Northwestern University	June 20, 1996	"Tumor supressor gene control of angiogenesis."

THE MOUNT SINAI MEDICAL CENTER
THE MOUNT SINAI HOSPITAL * MOUNT SINAI SCHOOL OF MEDICINE
MOUNT SINAI CANCER CENTER
BREAST CANCER STUDY GROUP
One Gustave L. Levy Place * Box 1130
New York, NY 10029

MEMORANDUM

April 30, 1996

FROM: Dr. Stuart Aaronson
Dr. Steven T. Brower

TO: **Distribution**

The next scheduled meeting for the Mount Sinai Cancer Center, Breast Cancer Study Group will be held on May 14, 1996 at 12:00 noon in the Annenberg Building, Room 25-51 on the 25th Floor.

SPEAKERS

***12:15 pm**

Dr. Jonathan Licht/ Assistant Professor of the Department of Medicine
Mount Sinai Medical Center

TOPIC: ***BRCA-1 Breast Cancer Suppressor Gene***

***12:45 pm**

Dr. Christine Eng/Assistant Professor of Human Genetics
Mount Sinai Medical Center

TOPIC: ***Issues in Breast Cancer Screening***

Any questions, please feel free to contact Mr. Angel Garcia at ext. 48026 or 212-241-8026.

LUNCH WILL BE SERVED

Thank you.

MOUNT SINAI CANCER CENTER BREAST STUDY GROUP
One Gustave L. Levy Place, Box # 1130
New York, NY 10029

MEMORANDUM

FROM: **Stuart Aaronson, M.D.**
Director
Derald Ruttenberg Cancer Center

Steven T. Brower, M.D.
Chief
Division of Surgical Oncology

TO: ***Distribution/Cancer Center Study Group***

The next Cancer Center Breast Study Group meeting will be held at 12:00 P.M. on July 27, 1995 in Conference Room Annenberg 25-51.

AGENDA

1. **New Breast Cancer Funding Opportunities**
   Dr. Stuart Aaronson
   Director, Derald Ruttenberg Cancer Center

2. **Student Research Projects for the Cancer Center Breast Study Group.**
   Max Fonarov-Student/J. Manfredi, MD-Preceptor
   Ullrich Hermanto-Student/L. Wang, MD-Preceptor
   Tara Santore-Student/Ravi Iyengar, MD-Preceptor

Should you have any questions, please feel free to call Mr. Angel Garcia at ext. 48026 or Mr. John Bazos at ext. 46470.

Thank you.

LUNCH WILL BE SERVED

APPENDIX F

BCPTP- TRAINEE AWARDS

1994-1995 Academic Year

	Stipend	Tuition & Fees	Supply Allowance	Total
Maxim Fonorev	14,833	2,947	350	18,130
Ulrich Hermanto	14,833	1,763	350	16,946
Tara Santore	14,833	882	350	16,065
Total	44,499	5,592	1,050	51,141

1995-1996 Academic Year

	Stipend	Tuition & Fees	Supply Allowance	Total
Jessica Feinleib	14,867	1,385	350	16,602
Maxim Fonorev	14,867	3,050	350	18,267
Ulrich Hermanto	14,867	1,385	350	16,602
Tara Santore	14,867	1,385	350	16,602
Wei Li	14,867	1,385	350	16,602
Total	74,335	8,590	1,750	84,675